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<p>(54) Title: INDUCTION OF CYTOLYTIC T-LYMPHOCYTES WITH CYTOMEGALOVIRUS POLYPEPTIDES</p> <p>(57) Abstract</p> <p>Immunogens for use in human cytomegalovirus vaccines or skin tests which comprise human cytomegalovirus matrix proteins or fractions thereof are disclosed. It is also disclosed that such matrix proteins are target antigens for human cytomegalovirus specific cytotoxic T-lymphocytes.</p>		

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Induction of Cytolytic T-lymphocytes with  
Cytomegalovirus Polypeptides

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RELATED APPLICATION

This application is a continuation-in-part of application Serial No. 07/307,526 filed February 8, 1989, which is a division of application Serial No. 06/885,386 filed July 16, 1986, which is a continuation of application Serial No. 06/635,368 filed July 27, 1984 and now abandoned.

FIELD OF THE INVENTION

This invention relates to human cytomegalovirus (HCMV) vaccines in which the immunogen is an HCMV matrix protein or a fragment of such a protein. This invention also relates to immunologic cytomegalovirus proteins or peptides that induce cytolytic T-lymphocytes effective to lyse cytomegalovirus infected cells, to the production of such proteins or peptides and to vaccines and skin tests in which such proteins or peptides are utilized.

ABBREVIATIONS

CMV	Cytomegalovirus
HCMV	Human Cytomegalovirus
CTL	Cytotoxic T-lymphocytes
MHC	Major Histocompatibility Complex

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IE	Immediate Early
gB	Glycoprotein B
CNBr	Cyanogen Bromide
HF	Human Foreskin Fibroblasts
FCS	Fetal Calf Serum
PBL	Peripheral Blood Lymphocytes
APC	Antigen Presenting Cell
ActD	Actinomycin D
pp65	An HCMV Matrix Protein--corresponds to "gp64" of Serial No. 07/307,526
TFA	Trifluoroacetic acid

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#### BACKGROUND OF THE INVENTION

Cytomegalovirus (CMV) infection is a frequent pathogen in humans. It is usually associated with asymptomatic primary infection, followed by a state of viral persistence or latency (1, 2). In patients with congenital or acquired immune deficiencies and those undergoing solid organ or bone marrow transplantation, primary CMV infection and reactivation of persistent CMV have frequently been associated with life-threatening invasive visceral disease (3, 5).

Application Serial No. 07/307,526 reports T-lymphocyte reactivity to the matrix protein of HCMV following natural infection of an individual. As that application explains, peripheral blood mononuclear leukocytes were separated using Ficoll-hypaque, washed, suspended in RPMI-1640 medium with ten percent (10%) AB+, Rh+ human serum and were then cultured at 37°C. in microtiter plates (10<sup>5</sup> cells/well). Different samples of the peripheral blood mononuclear leukocytes were then stimulated

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with optimal dilutions of individual ones of (a) the matrix protein of human cytomegalovirus (HCMVgp64) such as HCMVgp64, (b) HCMV-infected cell lysate and (c) control cell lysate, and cultures were analyzed for proliferation and for lymphokine production.

The matrix protein of human cytomegalovirus (4  $\mu$ g/ml) stimulated  $H^3$ -thymidine in HCMV seropositive donor peripheral blood mononuclear leukocytes but not in seronegative donor peripheral blood mononuclear leukocytes. The peripheral blood mononuclear leukocytes produced the same stimulation with HCMV-infected cell lysate as when the matrix protein of human cytomegalovirus (HCMVgp64) was used. The matrix protein of human cytomegalovirus (HCMVgp64) induced interleukin-2 (IL-2) production, IL-2 receptor expression and interferon production. The addition of antibody to the IL-2 receptor blocked the reactivity of the peripheral blood mononuclear leukocytes to HCMVgp64.

The development of cell-mediated immunity, particularly that involving CD8+ class I MHC-restricted CMV-specific CTL, represents an essential host factor in the control of persistent infection and the recovery from CMV disease (6, 9). The importance of CD8+ CTL responses in providing protective immunity in CMV seropositive individuals has fostered studies to elucidate the specificity of this response for selected CMV antigens with the objective of developing methods to augment CTL responses in immunocompromised hosts. Studies using vaccinia recombinant viruses to express the non-structural 72 KD CMV immediate early protein (IE) and the major envelope glycoprotein, gB, have demonstrated that in two CMV seropositive individuals, a subset of CTL recognized IE and a minor subset recognized gB (10). However, the

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majority of CMV-specific CTL in these individuals recognized other undefined viral antigens (10).

It is now established that CD8+ CTL recognize peptide fragments of antigens, derived as a result of intracellular processing of cytoplasmic protein and subsequently association with class I MHC molecules in the endoplasmic reticulum prior to cell surface presentation (12, 13, 14, 15). Consequently, small protein fragments or synthetic viral peptides can sensitize uninfected target cells by direct association with MHC molecules at the cell surface (16, 17, 18). Thus, an alternative approach to using recombinant expression vectors for examining the specificity of CTL recognition is to directly sensitize target cells by incubation with synthetic peptides or peptides derived from native protein by proteolytic digestion (19, 20).

#### DESCRIPTION OF THE FIGURES

Figure 1 demonstrates that polyclonal CMV-specific CTL lyse autologous cells from five different donors pulsed with CNBr digest of the CMV matrix protein pp65. CMV-specific CTL lines were generated from 5 CMV seropositive donors and assayed in a 4 hour chromium release assay for recognition of autologous target cells either infected for 24 hours with CMV (■), pulsed for 1 hour with CNBr cleaved pp65 (▲), or mock infected (●). Control MHC mismatched target cells were either infected for 24 hours with CMV (□), or pulsed for 1 hour with CNBr cleaved pp65 (Δ).

Figure 2 demonstrates in vitro stimulation of peripheral blood lymphocytes with CNBr cleaved pp65 elicits class I MHC restricted CTL that lyse CMV infected target cells. PBL from 4 donors were



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stimulated with CNBr cleaved pp65 either directly (donor MW) or presented on autologous fibroblasts (donors CM, MR, and DH). Effector cells from these cultures were assayed in a 4 hour chromium release assay for lysis of autologous fibroblast target cells either infected with CMV for 24 hours (■), pulsed for 1 hour with CNBr cleaved pp65 (▲), or mocked infected (●). Control MHC mismatched target cells infected with CMV for 24 hours (□) and pulsed with CNBr cleaved pp65 (Δ) were assayed to demonstrate that lysis is class I MHC restricted.

Figure 3 demonstrates pp65-specific CTL clones lyse CMV infected cells at all stages of the viral replicative cycle. CD3+, CD8+, CTL clones were derived from the pp65-specific CTL line generated from donor CM. Clones 1C8 (■) and 2C8 (▨) were assayed for recognition of autologous target cells either mock infected or infected with CMV at an MOI of 5 for 2 - 66 hours prior to use in a 4 hour chromium release assay. The effector to target ratio in this assay was 5:1. Lysis of MHC mismatched target cells infected with CMV was <5% at all time points (data not shown).

Figure 4 is a map of a pp65-CNBr digest.

#### SUMMARY OF THE INVENTION

This invention includes the discovery that HCMV matrix protein pp65 is a target antigen for HCMV specific CTL. CNBr cleavage of pp65 yields other CTL specific target antigens. Immunogens for use in HCMV vaccines or skin tests are provided by pp65 and by identification and synthesis of cytolytic pp65 sequences, e.g., the sequences 1 to 6 (Sequence ID Nos. 1-6), infra. An important advantage of this aspect of the invention is the simplicity and

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rapidity with which it can be determined whether an individual viral protein functions as a CTL target antigen without the construction of recombinant vectors. The need to synthesize substantial amounts of a large protein is avoided.

### EXPERIMENTAL

#### Virus and Cell Lines

CMV Towne strain was grown in human foreskin fibroblast (HF) monolayer cultures and harvested for virus antigen production 120 hours post infection as described by Clark, et al. (21). AD 169 strain CMV was obtained from ATCC and propagated by three serial passages onto confluent 150 cm<sup>2</sup> flasks of HF in DMEM supplemented with 2% FCS. Viral stocks were prepared from infectious supernatants when HF cells showed 100% cytopathic effect.

Primary dermal fibroblast lines were established by explant culture of a skin biopsy obtained using a standard procedure from 5 CMV seropositive individuals with the following HLA types: MW - A1, A24, B7, B8; CM - A1, A2, B35, B57; TM - A3, A24, B8, B51; MR - A24, A25, B18, B35; DH - A1, A31, B38, B44. These cells were maintained in DMEM or Waymouths media supplemented with 10-15% FCS (Hyclone Logan, UT), 2 mM L-glutamine, and penicillin/streptomycin. Fibroblasts were used as target cells after 4-12 passages. Bone marrow derived fibroblasts were obtained from a CMV seropositive transplant donor (SA) of HLA type A28, A30, B18, B35, and maintained in culture for 6 passages prior to use. EBV transformed B lymphoblastoid cell lines (LCL) were generated and maintained as previously described (22).

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CMV pp65 Purification and CNBr Cleavage

CMV pp65 was purified from extracellular virion plus dense bodies of CMV by reverse phase high performance liquid chromatography as previously described (23). Purity was assessed by amino acid analysis. For chemical cleavage purified pp65 was dissolved in 60% formic acid and treated with a 100 fold molar excess of CNBr (over methionine residues) overnight. The solvent was evaporated at room temperature overnight under a stream of argon, the residue resuspended in water and lyophilized.

Generation of Cytotoxic Effector Cells

CMV-specific polyclonal CTL were generated from the PBL of CMV seropositive, asymptomatic donors (CM, MR, TM, MW, and DH) using described methods (11, 22, 8). Briefly, PBL were isolated by Histopaque (Sigma, St. Louis, MO) gradient centrifugation and suspended in RPMI medium supplemented with 12% human CMV seronegative AB serum, 4 mM L-glutamine, 25  $\mu$ M 2 mercaptoethanol (Sigma), penicillin and streptomycin (referred to as culture media).  $1 \times 10^7$  PBL were cultured with  $5 \times 10^5$  autologous fibroblasts infected two hours previously with AD169 or Towne strain of CMV at an MOI of 5 in individual wells of a 6 well plate or in 25 cm<sup>2</sup> flasks in a final volume of 6 ml culture media. After 7 days, the cultures were restimulated with both autologous CMV-infected fibroblasts,  $\gamma$ -irradiated (3000 rads) autologous PBL, and then supplemented with recombinant IL2 (2-5 U/ml) 2 and 4 days after restimulation. After a total of 14 days in culture the effector cells were harvested and used in a standard Cr<sup>51</sup> release assay.

To generate pp65-specific CTL,  $5 \times 10^5$  autologous fibroblasts were cultured for 1 hour with 100  $\mu$ g/ml peptides from CNBr digested pp65 (CNBr pp65) in 0.2

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ml DMEM and used to stimulate  $1 \times 10^7$  PBL. These cultures were restimulated at 7 days with CNBr pp65 pulsed fibroblasts, and supplemented with IL2 (2-5 U/ml), 2 and 4 days following restimulation. After a total of 14 days in culture, the effector cells were harvested and used in a  $Cr^{51}$  release assay. For a single donor, PBL were cultured in the presence of 75  $\mu$ g/ml CNBr pp65 for 5 days and then assayed for CTL activity.

CD8+ pp65-specific CTL clones were generated from 14 day CTL lines by first depleting CD4+ T cells with OKT4 mAb (Ortho, Raritan, New Jersey) and rabbit complement (Pel freeze, Brown Deer, WI) and then plating the enriched CD8+ T cells in 96 well round bottom plates at 0.5 cells/well with autologous irradiated (5000 rads) LCL ( $2 \times 10^4$  cells/well) as feeder cells, and autologous CNBr pp65 pulsed fibroblasts ( $2 \times 10^3$  cells/well) as stimulators in 0.2 ml culture media with 50 U/ml of IL2. Wells positive for growth at 14 days were transferred to larger wells and the clones restimulated every 7 days with autologous CNBr pp65 pulsed fibroblasts or CMV-infected fibroblasts, supplemented with irradiated feeder cells and fed with IL2 (60 U/ml) 2 and 4 days following each restimulation.

#### Chromium Release Assay

A 4 hour chromium release assay was used to assay T cell lines and clones for cytolytic activity. Effector cells were harvested 5 to 7 days after restimulation, washed once in cold PBS and resuspended in RPMI with 10% FCS. Autologous and MHC-mismatched fibroblast target cells were infected with CMV at an MOI = 5 for 24 hours or incubated in the presence of CNBr-pp65 ( $1 \times 10^7$  cells/100  $\mu$ g of pp65) for 1 hour. In some experiments, BSA was used

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as a soluble protein control, and cells were treated as for CNBr pp65 cells. All target cells were then labelled with 200  $\mu$ Ci of  $\text{Cr}^{51}$  for 2 hours at 37°C.

Metabolic blockade with Actinomycin D (Act D) was used to prepare target cells such that they were infected with CMV but not endogenously synthesizing any CMV gene products, as previously described (11). Briefly,  $\text{Cr}^{51}$  labelled fibroblast monolayers were preincubated in medium containing 20  $\mu$ g/ml Act D for 30 minutes, infected with CMV (MOI of 5) in the continued presence of Act D for 4 hours, and maintained in Act D containing media during two washes and the 4 hour chromium release assay. In these experiments, control fibroblasts were infected with CMV for 4 hours in the absence of Act D.

For experiments examining the kinetics of pp65 presentation to CTL, fibroblasts were infected with CMV at an MOI = 5 for 2, 4, 12, 24, 36, 48 or 66 hours and labelled with  $\text{Cr}^{51}$  for 2 hours prior to use as target cells.

To prepare targets for plating, the  $\text{Cr}^{51}$  labelled fibroblasts were washed three times in cold HBSS and resuspended in RPMI 10% FCS for counting. For all assays, target cells were plated in triplicates in 96 well round bottom plates at  $1 \times 10^4$  cells/well, and effector cells were added to achieve various E/T ratios in a final volume of 200  $\mu$ l. Following a 4 hour incubation, plates were centrifuged at 70 x g for 10 minutes and 100  $\mu$ l of supernatant harvested for gamma counting. Spontaneous release was determined from target cells incubated with media alone, maximal release was determined from target cells incubated with 1% NP-40 (Sigma), and percent specific lysis was calculated using the standard formula. Spontaneous release was less than 15% for

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CMV-infected cells and less than 20% for CNBr-pp65 labelled cells.

#### Monoclonal Antibodies and Cell Surface Phenotype

Depletion of CD4 and CD8+ T cells from effector cell populations was done as previously described (11). T cell clones were phenotyped for CD3, CD4, and CD8 expression by indirect immunofluorescence using OKT3 ( $\alpha$ CD3), OKT4 ( $\alpha$ CD4) and OKT8 ( $\alpha$ CD8) mAb (Ortho, Raritan, New Jersey) and FITC conjugated goat antimouse Ig (Tago, Burlingame, CA).

#### Results

##### Recognition of pp65 by Polyclonal CD8+ CMV-specific CTL

To determine whether pp65 would serve as a significant target antigen for a component of the CMV-specific CTL derived from CMV seropositive individuals with protective immunity, polyclonal CMV-specific CTL were activated by stimulating peripheral blood lymphocytes with autologous CMV-infected fibroblasts in short term cultures. These effector cell populations were assayed for the ability to lyse autologous and MHC mismatched fibroblasts either infected with CMV, mock-infected, or pulsed with CNBr digested pp65. Polyclonal CMV-specific CTL lines generated from four of five consecutive donors studied lysed both autologous CNBr pp65 pulsed target cells and CMV-infected cells but not mock infected targets (Figure 1). Lysis of both CMV-infected and CNBr pp65 pulsed autologous targets was class I MHC-restricted since target cells that were mismatched for class I MHC antigens were not lysed (Figure 1). Lysis was predominantly mediated by CD8+ T cells since selective depletion of the CD8+ T cell subset but not the CD4+ subset abrogated lytic activity (Table 1).

Table 1

Lysis of autologous CMV-infected and CNBr pp65 pulsed fibroblasts by polyclonal CMV-specific CTL is mediated by CD8+ T cells

Cell Line	E/T*	mAb+C	Target Cell (% Lysis)					
			Autologous Target Cells			MHC Mismatched Target Cells		
			Mock	CMV	CNBr pp65	Mock	CMV	CNBr pp65
CMV	20:1	-	2	57	35	2	11	0
		$\alpha$ CD4	1	58	38	0	6	0
		$\alpha$ CD8	1	3	2	0	4	2
MRV	20:1	-	0	65	33	5	10	8
		$\alpha$ CD4	1	67	35	2	8	9
		$\alpha$ CD8	0	12	7	3	7	3
TMV	20:1	-	2	29	15	0	2	0
		$\alpha$ CD4	1	33	14	0	3	0
		$\alpha$ CD8	0	4	2	1	1	1

\* Effector - target ratio

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Fibroblasts pulsed with native pp65 were not lysed (data not shown). These results demonstrated that in the majority of CMV seropositive individuals a component of the memory CD8+ CMV-specific CTL response activated by stimulation with autologous CMV-infected fibroblasts, recognize epitopes of the CMV matrix protein pp65. In these individuals, the lytic profile of polyclonal CTL for CNBr pp65 pulsed autologous fibroblasts suggests that pp65-specific CTL comprise a significant subset of the CMV-specific CTL response. The inability to identify CTL that recognize CNBr pp65 pulsed fibroblasts in 1 of the 5 individuals may be due to an absence of a memory CTL response specific for pp65 in this individual or to a failure of CNBr cleavage of pp65 to produce peptides similar enough to the epitopes generated by intracellular antigen processing that are recognized in the context of class I MHC by CTL of this individual.

CD8+ pp65-Specific CTL Clones Recognize CMV-Infected Cells in the Absence of Viral Gene Expression

pp65 is one of the most abundant structural virion proteins (25), and thus represents an attractive candidate as a target antigen for this response. To determine if class I MHC restricted presentation of pp65 in CMV-infected cells was independent of viral gene expression, it was necessary to selectively isolate pp65-specific CTL and then determine whether those could lyse CMV infected target cells with and without viral gene blockage. The pp65 specific CTLs were isolated by culturing PBL directly with CNBr digested pp65 or by pulsing autologous  $\gamma$  irradiated fibroblasts with CNBr pp65 and using these cells as APC to stimulate PBL in short term cultures. In 3 of 4 donors, these



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stimulations consistently elicited CTL that lysed autologous CMV-infected or CNBr pp65 pulsed fibroblasts in a class I MHC-restricted fashion (Figure 2).

CD8+ pp65-specific T cell clones were then generated from one of these lines by plating CD8+ T cells in limiting dilution cultures with  $\gamma$  irradiated autologous CNBr pp65 pulsed fibroblasts as both stimulators and irradiated feeder cells. From these cultures, a panel of CD3+, CD8+, CD4- T cell clones were identified that lysed autologous CMV-infected and CNBr pp65 pulsed fibroblasts but not mock infected or MHC mismatched target cells. The cytolytic reactivity of 7 representative clones is shown in Table 2.

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Table 2

Cytolytic reactivity of seven representative  
CD8+ T cell clones specific for the  
CMV matrix protein pp65

Clone	E/T*	Target Cell (% Lysis)			Autologous Cells			MHC Mismatched Cells		
		Mock	CMV	CNBr pp65	Mock	CMV	CNBr pp65	Mock	CMV	CNBr pp65
CM1C8	5:1	0	30	57	0	0	0	0	0	0
CM11D3	5:1	1	37	74	1	0	1	0	0	1
CM15F11	5:1	1	33	71	0	1	1	0	1	1
CM2C8	5:1	0	30	57	0	0	0	0	0	0
CM16E6	5:1	1	31	70	1	2	1	1	2	1
CM8A10	5:1	0	33	60	0	0	0	0	0	0
CM6H12	5:1	2	32	74	1	2	1	1	2	1

\* Effector/Target ratio.

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The pp65-specific T cell clones were then assayed for the ability to lyse autologous fibroblasts infected with CMV in the presence of the RNA synthesis inhibitor ActD. The concentration of ActD used in these experiments has previously been shown to block detectable CMV gene transcription in infected cells (11). The pp65-specific T cell clones lysed autologous ActD blocked CMV-infected target cells as efficiently as target cells infected in the absence of ActD (Table 3), demonstrating that this CMV matrix protein is available for processing and presentation for CTL recognition without the requirement for viral protein synthesis following viral entry.

Table 3

CD8+ pp65-Specific CTL Clones Recognize  
CMV-Infected Target Cells in The Absence  
of Viral Gene Expression

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Clone	TARGET CELL (% Lysis)				MHC Mismatched Cells			
	Autologous Cells							
	Mock	CMV	CMV/ActD	CNBrpp65	Mock	CMV	CMV/ActD	CNBrpp65
11D3	0/1/0	18/21/27	23/24/28	71/74/80	2/2/2	2/2/0	0/0/0	0/0/0
15C10	2/2/3	14/16/21	19/24/27	59/75/83	3/3/2	0/0/1	0/1/1	1/0/0
15F2	2/2/2	20/22/24	22/25/28	71/74/81	1/2/2	0/0/1	1/0/0	0/0/0
15F11	0/1/0	14/16/28	10/17/23	60/71/77	1/2/2	0/0/1	1/0/0	0/0/0
16E6	2/1/0	12/21/24	20/21/22	60/73/75	2/2/1	0/0/0	1/1/1/	2/0/0

CD8+ pp65-specific T cell clones generated from CMV seropositive donor CM (HLA A1, A2, B35, B57) as described in Table 2 were assayed for recognition of autologous and MHC mismatched fibroblast target cells either mock infected, infected with CMV for 4 hours, pretreated with ActD and infected with CMV for 4 hours or pulsed with CNBr pp65 for 1 hour. The numbers represent specific lysis of target cells at E/T ratios of 1.25:1/2.5:1/5:1.

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pp65-Specific CTL Recognize CMV-Infected Cells at all Stages of The Viral Replicative Cycle

To determine if pp65 is efficiently presented by CMV-infected cells throughout the replicative cycle, the recognition of autologous cells infected with CMV for various periods of time (2-66 hours) prior to use as targets in the chromium release assay was examined. These experiments demonstrated that pp65-specific T cell clones recognized target cells infected with CMV at all stages of the viral replicative cycle. Representative data is shown for two clones in Figure 3. Lysis of CMV-infected cells by these clones is evident at the earliest time point (2 hours) after infection, and peaks at 36 and 48 hours after infection. Thus, presentation of the pp65 matrix protein in CMV infected cells for class I MHC restricted T cell recognition occurs promptly after viral entry and during all stages of the viral replicative cycle.

The structural matrix protein pp65 has been identified as a target antigen for CMV-specific class I MHC restricted CTL derived from the peripheral blood of 4 of 5 asymptomatic CMV seropositive individuals. The lytic profile observed for polyclonal CMV-specific CTL on CNBr pp65 sensitized target cells suggests that pp65 represents a significant target antigen, among others, recognized by CMV-specific CTL. The described method to identify pp65 specific CTL is based on the many observations that synthetic peptides and chemically or enzymatically cleaved protein fragments can substitute for endogenously expressed protein antigens when presented to CTL in the context of MHC on an appropriate target cell (12, 16, 17, 18, 19, 20). This approach circumvents the need to generate

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recombinant viral vectors expressing single CMV genes when the putative antigen can be obtained as a soluble protein. Synthetic viral peptide fragments may sensitize uninfected cells for lysis either by directly associating with class I molecules, thus bypassing the cellular processing requirement altogether, or by being internalized by the cell in an already degraded form that enables the peptide to enter the intracellular trafficking and processing pathways leading to class I MHC association and presentation (33). In either case, the result is the presence at the cell surface of class I MHC peptide complexes of sufficient density for recognition by CTL. The CNBr-pp65 digest used in the described experiments apparently works in a similar manner, except that several protein fragments are present, one or more of which would be similar to a naturally processed fragment produced in CMV-infected cells. This is consistent with observations that antigen processing events for native proteins include internalization and degradation within lysosomal compartments resulting in association with class II and not class I MHC molecules for transport to the cell surface (34). The advantage of this aspect of the invention is the simplicity and rapidity with which it can be determined whether an individual virion protein is able to serve as a CTL target antigen without the construction of recombinant vectors. The disadvantages are that not all viral proteins can be readily purified from infected cells, and chemical or enzymatic cleavage may not produce protein fragments capable of mimicking all the epitopes produced by endogenous processing in the virally infected cell.

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This invention also demonstrates that CD8+ class I MHC restricted CTL specific for the matrix protein pp65, are readily isolated from the majority of healthy CMV seropositive individuals. These CTL will recognize CMV-infected cells without the requirement for viral gene expression and thus may provide an important effector cell for limiting CMV reactivation as well as for the resolution of active CMV disease.

A strong CTL response to virion proteins such as the pp65 matrix protein which are available for class I antigen processing immediately following virion entry and prior to the onset of viral replication in the infected cell is biologically attractive since such a CTL response may be particularly effective at limiting the potential for viral reactivation to progress to clinical disease. If the pp65-specific CTL response is also a major contributor to the resolution of active CMV infection, it would be expected that CMV-infected cells would be targets for CTL throughout the duration of the viral replicative cycle. Following CMV entry and uncoating, the viral genome is expressed in a cascade fashion with sequential expression of immediate early (0-2 hours), early (2-24 hours) and late (>24 hours) viral proteins (26). Abundant expression of pp65 does not occur until late stages of viral replication (27). Nevertheless, analysis of CTL induced target cell lysis after CMV infection indicated that pp65 specific lysis was present at all times post infection with a peak activity at 36-48 hours consistent with peak synthesis of this protein (25).

Studies in immunocompromised bone marrow transplant patients have identified that recovery of CD8+ CMV-specific CTL responses correlates with protection from developing CMV disease, but that

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greater than 50% of BMT recipients have delayed recovery of, or absent CMV-specific CTL responses for prolonged periods of time following transplant. This group of patients is at particularly high risk for developing CMV pneumonia (9). Thus, the identification of specific CTL target antigens provided by this invention is useful in the design of methods to augment CMV-specific immunity in BMT recipients. Synthetic viral peptides have recently been shown to be effective in specifically priming naive animals to generate high affinity virus-specific CTL when the peptide is conjugated to a lipoprotein or contained within a liposome (36, 37). Such an approach has the advantage of potentially eliciting CTL responses to CMV in immunosuppressed patients without the requirement for exposure to infectious virus. The demonstration that pp65 is a target antigen for CTL in the majority of seropositive individuals throughout the viral replicative cycle suggests that pp65 is an appropriate antigen to target in efforts to provide immunocompromised hosts with immunity to CMV.

Isolation and Purification  
pp65-CNBr Digest Peptides

This aspect of the invention includes the preparation of CNBr digests of pp65 and the isolation and purification of immunologically significant peptides from such digests.

For chemical cleavage with CNBr, pp65 was dissolved in 60% formic acid and treated overnight under argon with a 100 fold molar excess of CNBr (over methionine residues). The solvent was evaporated at room temperature under a stream of argon, the residue resuspended in water and lyophilized. The peptides were fractionated by HPLC



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on a Vydac C4 reverse phase column, using a 90-minute linear gradient from 100% solvent A (0.1% TFA) to 100% solvent B (TFA/H<sub>2</sub>O/CH<sub>3</sub>CN, 0.1/9.9/90, v/v/v). The HPLC purified peaks from map A were subjected to amino acid sequencing (performed by City of Hope) and from map B were used for CTL analysis (performed at the Fred Hutchinson Cancer Center).

Figure 4 is a map produced by the HPLC analysis. The HPLC purified peptide peaks designated by asterisks in Figure 4 were sequenced. These peptides correspond to sequences 1 through 6 (Sequence ID Nos. 1-6), of this application. The invention includes the use of these six peptides or portions thereof to lyse HCMV infected cells, and as immunogens in HCMV vaccines and skin test formulations.

-24-

SEQUENCE LISTING

## (1) GENERAL INFORMATION:

(i) APPLICANT: Pande, Hema  
Riggs, Arthur  
Zaia, John A.

## (ii) TITLE OF INVENTION:

Induction of Cytolytic T-Lymphocytes With  
Cytomegalovirus Proteins and Peptides

(iii) NUMBER OF SEQUENCES: Six

## (iv) CORRESPONDENCE ADDRESS:

(A) ADDRESSEE: City of Hope  
(B) STREET: 1500 East Duarte Road  
(C) CITY: Duarte  
(D) STATE: California  
(E) COUNTRY: United States of America  
(F) ZIP: 91010-0269

## (v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: 3M Double Density  
5 1/4" diskette  
(B) COMPUTER: Wang PC  
(C) OPERATING SYSTEM: MS DOS Version 3.20  
(D) SOFTWARE: Microsoft

## (vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER: Unknown  
(B) FILING DATE:  
(C) CLASSIFICATION: Unknown

## (vii) PRIOR APPLICATION DATA:

(A) APPLICATION NUMBER: US 07/307,526

-25-

(B) FILING DATE: 8 February 1989

## (viii) ATTORNEY/AGENT INFORMATION:

(A) NAME: Irons, Edward S.

(B) REGISTRATION NUMBER: 16,541

(C) REFERENCE/DOCKET NUMBER: None

## (ix) TELECOMMUNICATION INFORMATION:

(A) TELEPHONE: (202) 785-6938

(B) TELEFAX: (202) 785-5351

(C) TELEX: 440087 LM WSH

## (2) INFORMATION FOR SEQ ID NO:1

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 98 amino acid residues

(B) TYPE: Amino Acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Unknown

## (ii) MOLECULE TYPE:

(A) DESCRIPTION: Fragment of pp65 protein expressed by HCMV (Towne)

(iii) HYPOTHETICAL: No

(iv) ANTI-SENSE: Not applicable

(v) FRAGMENT TYPE: Internal Fragment

## (vi) ORIGINAL SOURCE:

(A) ORGANISM: HCMV

(B) STRAIN: Towne

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## (C) INDIVIDUAL ISOLATE

HCMV (Towne strain) was obtained from S. Star (Childrens Hospital, Philadelphia) (39) and passaged for <10 times in the lab. The resulting virus was used as inoculum.

(vii) IMMEDIATE SOURCE: None

(viii) POSITION IN GENOME: Not applicable

## (ix) FEATURE:

(A) NAME/KEY: Induces CTLs that lyse HCMV infected cells

## (B) LOCATION:

The sequence of pp65 corresponds to nucleotides (121037-119355) of the HCMV (AD169) sequence by Chee, et al. in (HCMV sequence in EMBL Release 23, May 1990 accession no. X17403). The protein pp65 is 561 amino acid in size. The peptide sequence ID No. 1 corresponds to amino acid 12-109 in this protein.

(C) IDENTIFICATION METHOD: By experiment

(D) OTHER INFORMATION: Induces CTLs that lyse HCMV infected cells

(x) PUBLICATION INFORMATION: None

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1

ISVLGPISGH VLKAVFSRGD TPVLPHETRL LQTFIHVRVS QPSLILVSQY  
TPDSTPCHRG DNQLQVQHTY FTGSEVENVS VNVHNPTGRS ICPSQPEPM

## INFORMATION FOR SEQ ID NO:2

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 11 amino acid residues

(B) TYPE: Amino Acid

(C) STRANDEDNESS: Single

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- (D) TOPOLOGY: Unknown
- (ii) MOLECULE TYPE:
- (A) DESCRIPTION: Fragment of HCMV pp65 protein
- (iii) HYPOTHETICAL: No
- (iv) ANTI-SENSE: Not available
- (v) FRAGMENT TYPE: Internal Fragment
- (vi) ORIGINAL SOURCE:
- (A) ORGANISM: HCMV
- (B) STRAIN: Towne
- (C) INDIVIDUAL ISOLATE:
- HCMV (Towne strain) was obtained from S. Star (Childrens Hospital, Philadelphia) (39) and passaged for <10 times in the lab. The resulting virus was used as inoculum.
- (vii) IMMEDIATE SOURCE: Not available
- (viii) POSITION IN GENOME: Not available
- (ix) FEATURE:
- (A) NAME/KEY: Induces CTLs that lyse HCMV infected cells
- (B) LOCATION:
- The sequence of pp65 corresponds to nucleotides (121037-119355) of the HCMV (AD169) sequence by Chee, et al. in (HCMV sequence in EMBL Release 23, May 1990 accession no. X17403). The protein pp65 is 561 amino acid in size. The peptide sequence ID No. 1 corresponds to amino acid 110-120 in this protein.
- (C) IDENTIFICATION METHOD: By experiment
- (D) OTHER INFORMATION: Induces CTLs that lyse HCMV infected cells
- (x) PUBLICATION INFORMATION: None

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2

S I Y V Y A L P L K M

## INFORMATION FOR SEQ ID NO:3

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 33 amino acid residues
- (B) TYPE: Amino Acid
- (C) STRANDEDNESS: Single
- (D) TOPOLOGY: Unknown

## (ii) MOLECULE TYPE:

- (A) DESCRIPTION: Fragment of HCMV pp65 protein

## (iii) HYPOTHETICAL: No

## (iv) ANTI-SENSE: Not available

## (v) FRAGMENT TYPE: Internal Fragment

## (vi) ORIGINAL SOURCE:

- (A) ORGANISM: HCMV
- (B) STRAIN: Towne
- (C) INDIVIDUAL ISOLATE

HCMV (Towne strain) was obtained from S. Star (Childrens Hospital, Philadelphia) (39) and passaged for <10 times in the lab. The resulting virus was used as inoculum.

## (vii) IMMEDIATE SOURCE: Not available

## (viii) POSITION IN GENOME: Not applicable

## (ix) FEATURE:

- (A) NAME/KEY: Induces CTLs that lyse HCMV infected cells

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## (B) LOCATION:

The sequence of pp65 corresponds to nucleotides (121037-119355) of the HCMV (AD169) sequence by Chee, et al. in (HCMV sequence in EMBL Release 23, May 1990 accession no. X17403). The protein pp65 is 561 amino acid in size. The peptide sequence ID No. 1 corresponds to amino acid 176-208 in this protein.

(C) IDENTIFICATION METHOD: By experiment

(D) OTHER INFORMATION: Induces CTLs that lyse HCMV infected cells

(x) PUBLICATION INFORMATION: None

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3

KEPDVYYTSA FVFPTKDVAL RHVCAHELV CSM

## INFORMATION FOR SEQ ID NO:4

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 8 amino acid residues

(B) TYPE: Amino Acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Unknown

## (ii) MOLECULE TYPE:

(A) DESCRIPTION: Fragment of HCMV pp65 protein

(iii) HYPOTHETICAL: No

(iv) ANTI-SENSE: Not applicable

(v) FRAGMENT TYPE: Internal Fragment

## (vi) ORIGINAL SOURCE:

(A) ORGANISM: HCMV

(B) STRAIN: Towne

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## (C) INDIVIDUAL ISOLATE

HCMV (Towne strain) was obtained from S. Star (Childrens Hospital, Philadelphia) (39) and passaged for <10 times in the lab. The resulting virus was used as inoculum.

(vii) IMMEDIATE SOURCE: Not available

(viii) POSITION IN GENOME: Not Applicable

## (ix) FEATURE:

(A) NAME/KEY: Induces CTLs that lyse HCMV infected cells

## (B) LOCATION:

The sequence of pp65 corresponds to nucleotides (121037-119355) of the HCMV (AD169) sequence by Chee, et al. in (HCMV sequence in EMBL Release 23, May 1990 accession no. X17403). The protein pp65 is 561 amino acid in size. The peptide sequence ID No. 1 corresponds to amino acid 209-216 in this protein.

(C) IDENTIFICATION METHOD: By experiment

(D) OTHER INFORMATION: Induces CTLs that lyse HCMV infected cells

(x) PUBLICATION INFORMATION: None

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4

E N T R A T K M

## INFORMATION FOR SEQ ID NO:5

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 15 amino acid residues

(B) TYPE: Amino Acid

(C) STRANDEDNESS: Single



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- (D) TOPOLOGY: Unknown
- (ii) MOLECULE TYPE:
  - (A) DESCRIPTION: Fragment of HCMV pp65 protein
- (iii) HYPOTHETICAL: No
- (iv) ANTI-SENSE: Not available
- (v) FRAGMENT TYPE: Internal Fragment
- (vi) ORIGINAL SOURCE:
  - (A) ORGANISM: HCMV
  - (B) STRAIN: Towne
  - (C) INDIVIDUAL ISOLATE

HCMV (Towne strain) was obtained from S. Star (Childrens Hospital, Philadelphia) (39) and passaged for <10 times in the lab. The resulting virus was used as inoculum.
- (vii) IMMEDIATE SOURCE: Not available
- (viii) POSITION IN GENOME: Not Applicable
- (ix) FEATURE:
  - (A) NAME/KEY: Induces CTLs that lyse HCMV infected cells
  - (B) LOCATION:

The sequence of pp65 corresponds to nucleotides (121037-119355) of the HCMV (AD169) sequence by Chee, et al. in (HCMV sequence in EMBL Release 23, May 1990 accession no. X17403). The protein pp65 is 561 amino acid in size. The peptide sequence ID No. 1 corresponds to amino acid 485-499 in this protein.
  - (C) IDENTIFICATION METHOD: By experiment
  - (D) OTHER INFORMATION: Induces CTLs that lyse HCMV infected cells

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- (x) PUBLICATION INFORMATION: None
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:5
- P P W Q A G I L A R N L V P M

## INFORMATION FOR SEQ ID NO:6

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 62 amino acid residues
  - (B) TYPE: Amino Acid
  - (C) STRANDEDNESS: Single
  - (D) TOPOLOGY: Unknown
- (ii) MOLECULE TYPE:
- (A) DESCRIPTION: Fragment of HCMV pp65 protein
- (iii) HYPOTHETICAL: No
- (iv) ANTI-SENSE: Not available
- (v) FRAGMENT TYPE: C-terminal fragment
- (vi) ORIGINAL SOURCE:
- (A) ORGANISM: HCMV
  - (B) STRAIN: Towne
  - (C) INDIVIDUAL ISOLATE
- HCMV (Towne strain) was obtained from S. Star (Childrens Hospital, Philadelphia) (39) and passaged for <10 times in the lab. The resulting virus was used as inoculum.
- (vii) IMMEDIATE SOURCE: Not available
- (viii) POSITION IN GENOME: Not Applicable
- (ix) FEATURE:
- (A) NAME/KEY: Induces CTLs that lyse HCMV infected cells

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## (B) LOCATION:

The sequence of pp65 corresponds to nucleotides (121037-119355) of the HCMV (AD169) sequence by Chee, et al. in (HCMV sequence in EMBL Release 23, May 1990 accession no. X17403). The protein pp65 is 561 amino acid in size. The peptide sequence ID No. 1 corresponds to amino acid 500-561 in this protein.

## (C) IDENTIFICATION METHOD: By experiment

## (D) OTHER INFORMATION: Induces CTLs that lyse HCMV infected cells

## (x) PUBLICATION INFORMATION: None

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:6

VATVQGQNLK YQEFFWDAND IYRIFAELEG VWQPAAQPKR RRHRQDALPG  
PCIASTPKKH RG

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## CLAIMS:

1. Cytotoxic T lymphocytes that lyse HCMV infected cells.
2. A method which comprises inducing CTLs by subjecting T cells to pp65 or to an immunologically effective fragment of pp65.
3. An isolated, purified peptide having Sequence ID NO. 1, or ID NO. 2, or ID NO. 3, or ID NO. 4, or ID NO. 5 or or ID NO. 6.
4. A man made peptide having Sequence ID NO. 1, or ID NO. 2, or ID NO. 3, or ID NO. 4, or ID NO. 5 or or ID NO. 6.
5. A method which comprises lysing HCMV infected cells by exposing said cells to pp65 or to an immunologically effective fragment of pp65.
6. A method as defined by claim 5 in which said fragment of pp65 is present in CNBr digest of pp65.
7. A method which comprises lysing HCMV infected cells by exposing said cells to pp65 or to a peptide of Sequence ID NO. 1, or ID NO. 2, or ID NO. 3, or ID NO. 4, or ID NO. 5 or or ID NO. 6.
8. A human cytomegalovirus vaccine in which the immunogen is a peptide have Sequence ID NO. 1.
9. A human cytomegalovirus vaccine in which the immunogen is a peptide have Sequence ID NO. 2.
10. A human cytomegalovirus vaccine in which the immunogen is a peptide have Sequence ID NO. 3.
11. A human cytomegalovirus vaccine in which the immunogen is a peptide have Sequence ID NO. 4.
12. A human cytomegalovirus vaccine in which the immunogen is a peptide have Sequence ID NO. 5.
13. A human cytomegalovirus vaccine in which the immunogen is a peptide have Sequence ID NO. 6.
14. A human CMV vaccine in which the immunogen is a mixture of peptides with Sequence ID NOS. 1-6.

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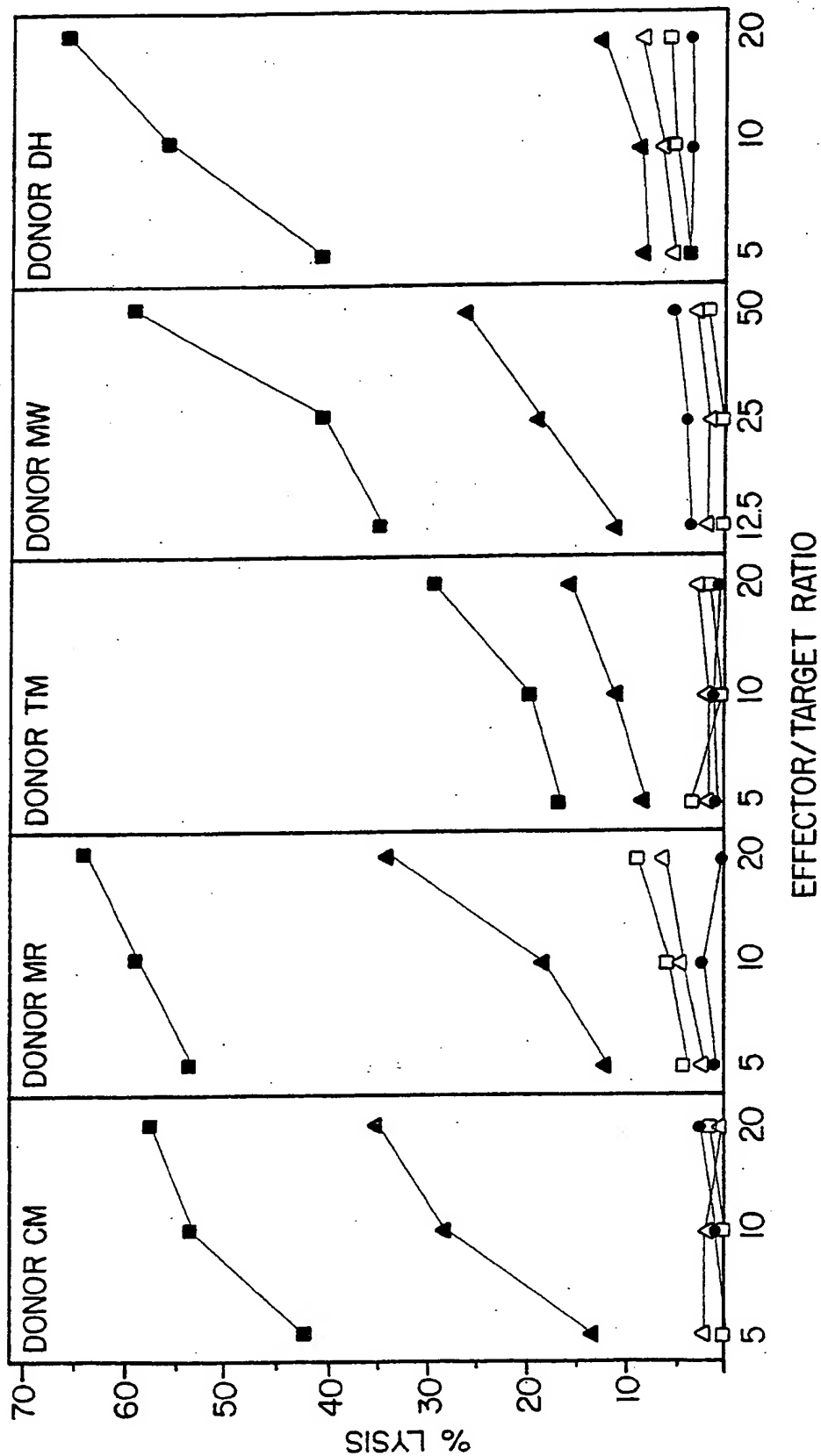
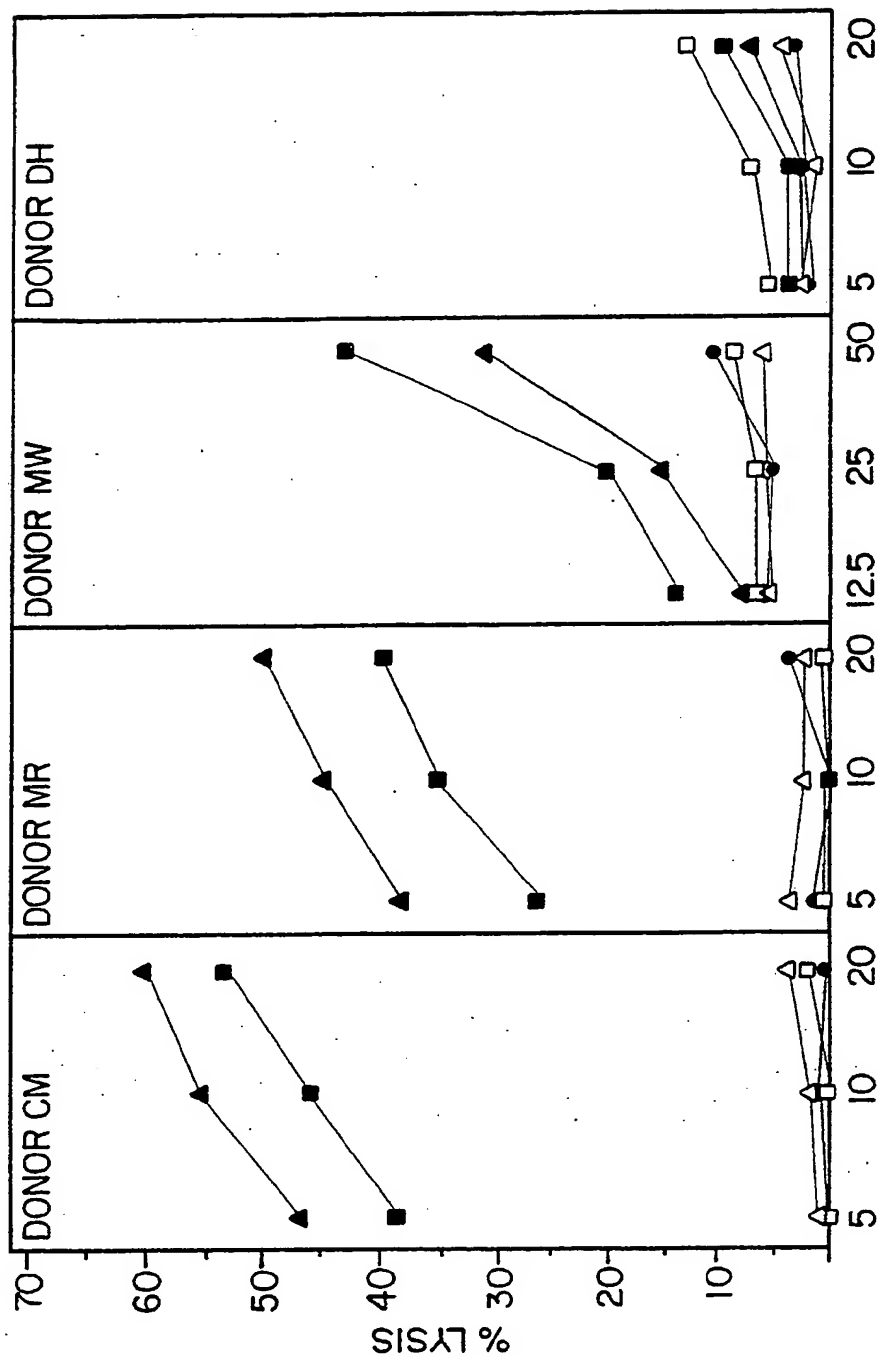


FIG. 1

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EFFECTOR/TARGET RATIO

FIG. 2

SUBSTITUTE SHEET

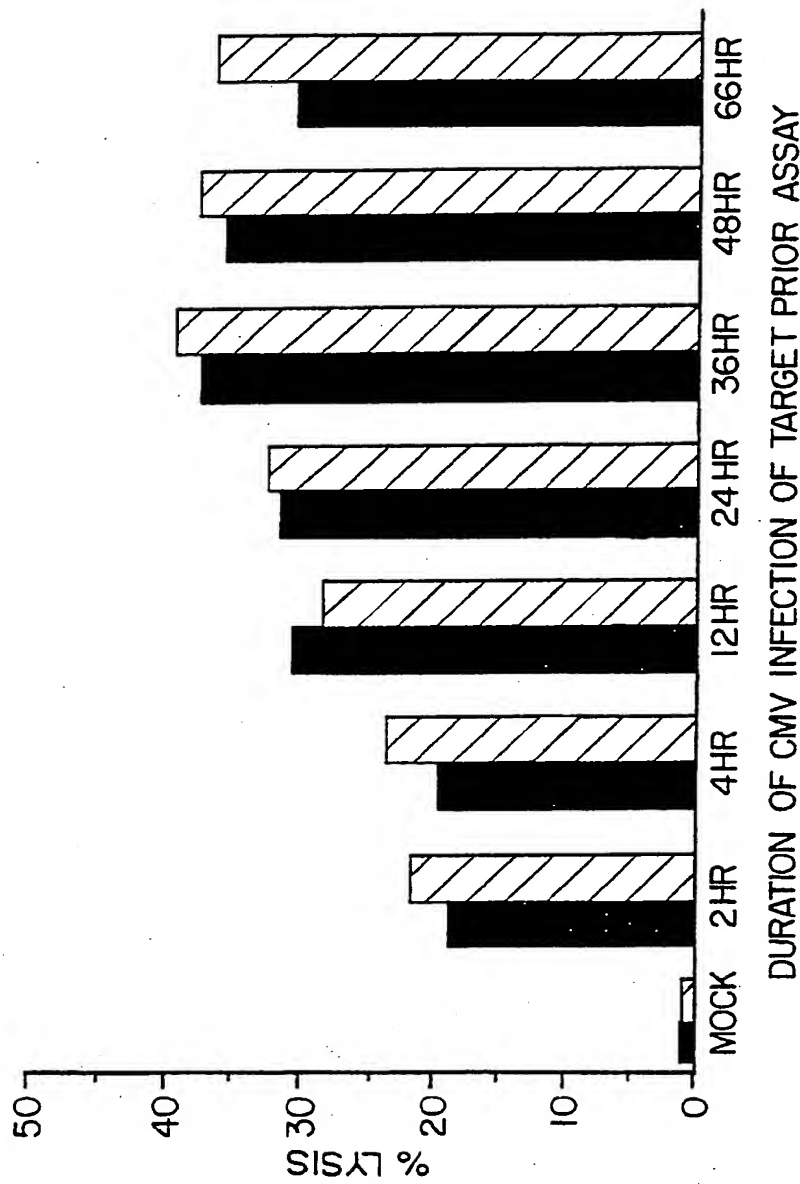


FIG. 3

SUBSTITUTE SHEET

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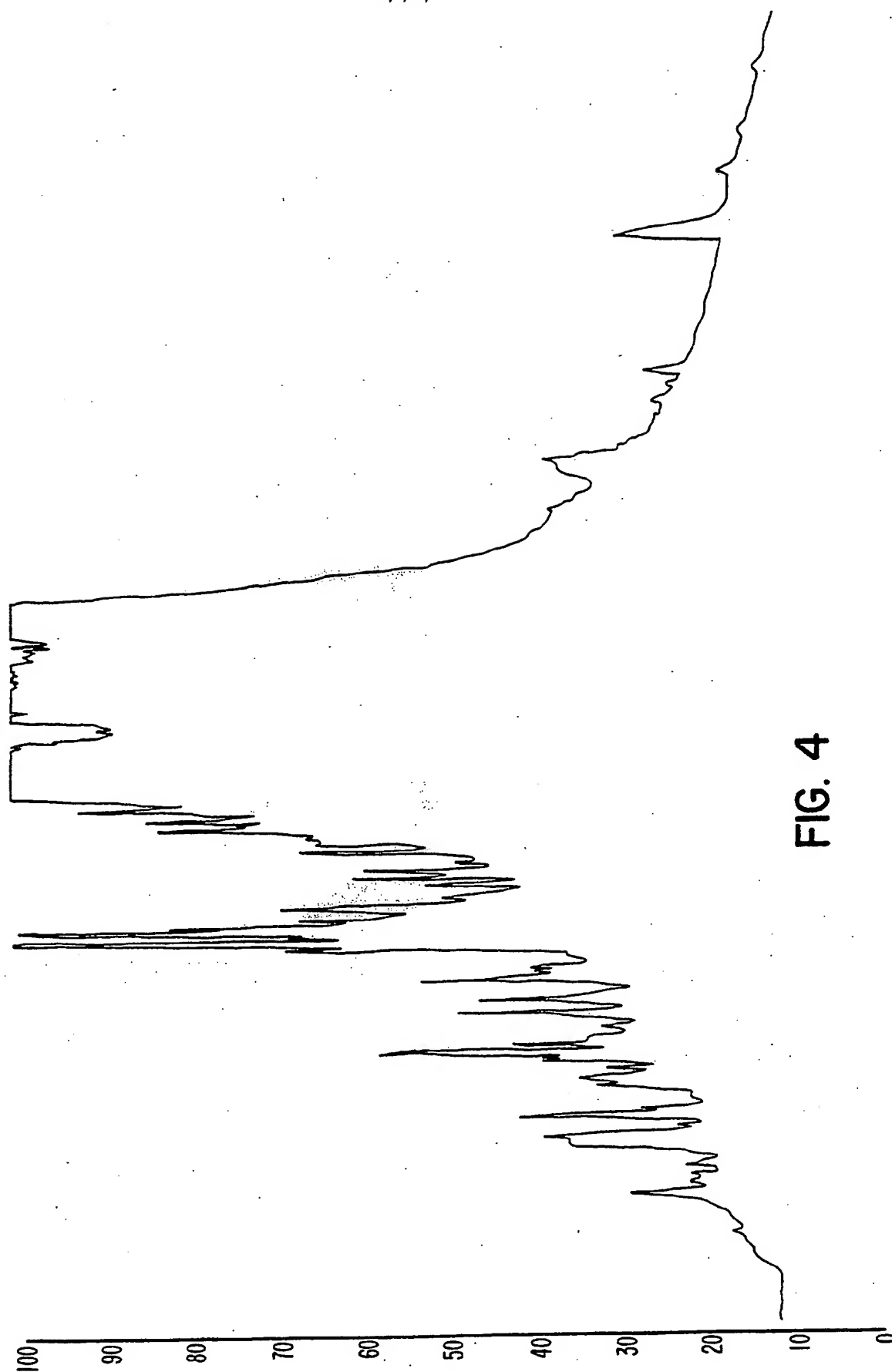


FIG. 4

SUBSTITUTE SHEET



## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US92/05432

## A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) : A61K 39/12; C07K 7/00, 13/00, 15/00; C12N 5/06

US CL : 424/89; 435/240.2; 514/12, 14, 15, 16; 530/324, 326, 327, 328, 350

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/89; 435/240.2; 514/12, 14, 15, 16; 530/324, 326, 327, 328, 350

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Automated Patent Searching, MEDLINE, CAS ONLINE, BIOSIS, WORLD PATENTS INDEX

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	European Journal of Immunology, Volume 13, No. 10, issued October 1983, L. K. Borysiewicz et al, "Human Cytomegalovirus-specific Cytotoxic T-lymphocytes: Requirements for in vitro Generation and Specificity", pages 804-809, especially the Abstract.	1-2, 5-7
Y	Journal of Virology, Volume 49, No. 1, issued January 1984, B. R. Clark et al, "Isolation and Partial Chemical Characterization of a 64,000-Dalton Glycoprotein of Human Cytomegalovirus", pages 279-282, see entire document.	1-14
Y	Virology, Volume 129, issued August 1983, D. Weiner et al, "Phosphorylation, Maturation Processing, and Relatedness of Strain Colburn Matrix Proteins", pages 155-169, see the Abstract.	1-14

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	* T	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
* A		document defining the general state of the art which is not considered to be part of particular relevance
* E	* X	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
* L	* Y	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
* O		document referring to an oral disclosure, use, exhibition or other means
* P	* G	document published prior to the international filing date but later than the priority date claimed

Date of the actual completion of the international search

17 August 1992

Date of mailing of the international search report

24 AUG 1992

Name and mailing address of the ISA/  
Commissioner of Patents and Trademarks  
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Washington, D.C. 20231

Facsimile No. NOT APPLICABLE

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Form PCT/ISA/210 (second sheet)(July 1992)\*

## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US92/05432

## C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	The Journal of Immunology, Volume 127, No. 3, issued September 1981, M. A. N. Wabuke-Bunoti et al, "Stimulation of Anti-influenza Cytolytic T Lymphocytes by CNBr Cleavage Fragments of the Viral Hemagglutinin", pages 1122-1125, especially the Abstract and Fig. 3.	2-14
Y	The Journal of Immunology, Volume 131, No. 5, issued November 1983, L. R. Gooding et al, "Recognition by Cytotoxic T Lymphocytes of Cells Expressing Fragments of the SV40 Tumor Antigen", pages 2580-2586, especially the Abstract.	2-14
Y	The Journal of Immunology, Volume 134, No. 5, issued May 1985, S. T. Forman et al, A 64,000 Dalton Matrix Protein of Human Cytomegalovirus Induces in vitro Immune Responses similar to Those of Whole Viral Antigen", pages 3391-3395, especially the Abstract.	1-14
X	US, A, 5,075,213 (Pande et al) 24 December 1991, entire document.	1-2, 5-7
Y		3-4, 8-14
Y	US, A, 4,058,598 (Stern et al) 15 November 1977, entire document.	8-14